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Pulsed-Field Gel Electrophoresis

Mary Elizabeth Kaufmann

1. Introduction

Pulsed-field gel electrophoresis (PFGE) was first described by Schwartz and Cantor *(1)* It is now an umbrella term for the alternating of an electric field in more than one direction through a solid matrix to achieve the separation of DNA fragments. The method requires the preparation of unsheared DNA, digestion of the DNA using a rare-cutting restriction endonuclease, separation of fragments by PFGE, and the visualization and interpretation of banding patterns (**Fig. 1**). Conventional agarose gel electrophoresis employs a static field and can resolve DNA fragments up to 50 kilobases (kb), although in practice, fragments larger than 20 kb co-migrate under the conditions usually applied. By introducing a pulse or change in the direction of the electric field, fragments as large as 10 megabases (Mb) can be separated. The time required by DNA fragments of different sizes to reorientate to the new electric field is proportional to their molecular weight and it is this factor that allows the separation and focusing of DNA fragments.

The main applications of PFGE in medical microbiology are as described in Chapter 2. With the implication in infections of an increasing variety of species for which phenotypic typing schemes are not available, PFGE provides a potentially universal method for fingerprinting and comparing isolates. However, it is not a typing method as, with the ability to produce an infinite variety of banding patterns, a PFGE profile can rarely be used to identify a documented bacterial strain, particularly when the technique is performed in different laboratories. Despite this, as a means of comparing isolates to determine whether they are the same or different, PFGE is arguably the most discriminating method currently available *(2)*.

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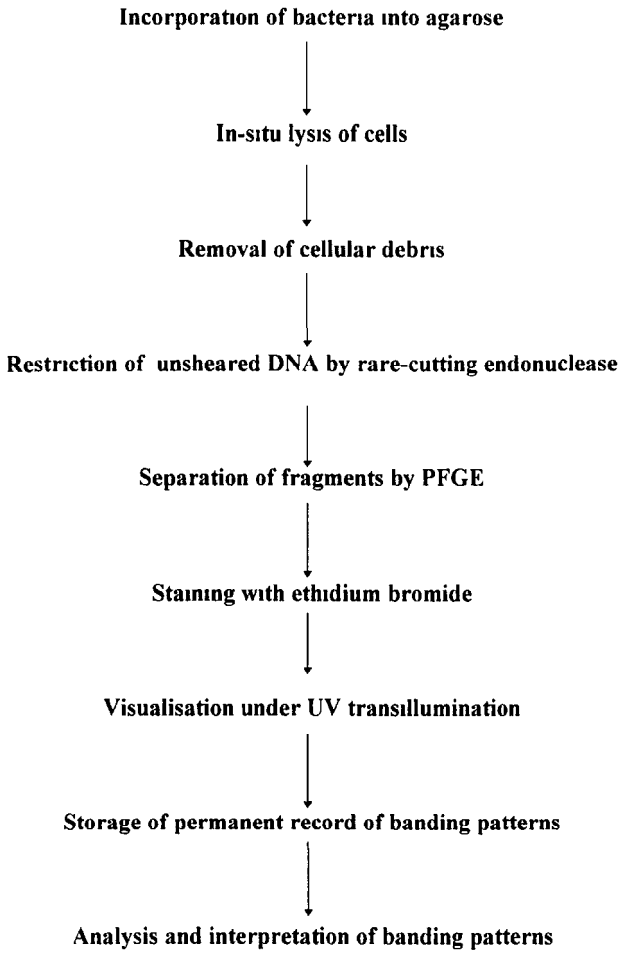
Incorporation of bacteria into agarose
In-situ lysis of cells
Removal of cellular debris
Restriction of unsheared DNA by rare-cutting endonuclease
Separation of fragments by PFGE
Staining with ethidium bromide
Visualisation under UV transillumination
Storage of permanent record of banding patterns
Analysis and interpretation of banding patterns

Fig 1. Practical steps involved in PFGE.

The most commonly used equipment is of two types; in one type the electric fields pulse forwards and backwards through the gel as in field-inversion gel electrophoresis (FIGE) *(3)*, whereas the other employs the contour-clamped homogeneous electric field (CHEF) *(4)*. FIGE equipment is simple, using a conventional electrophoresis tank, powerpack, and a switching unit. To achieve net forward migration in FIGE, the forward pulse time is longer than the backward pulse time, usually in a constant ratio. A development of FIGE is zero-integrated field electrophoresis (ZIFE) in which the forward voltage gradient is higher than the reverse, but the product of voltage and time is only slightly higher in the forward direction. As the difference in the two fields is only small, long run times are required, but the resulting bands are of high resolution.

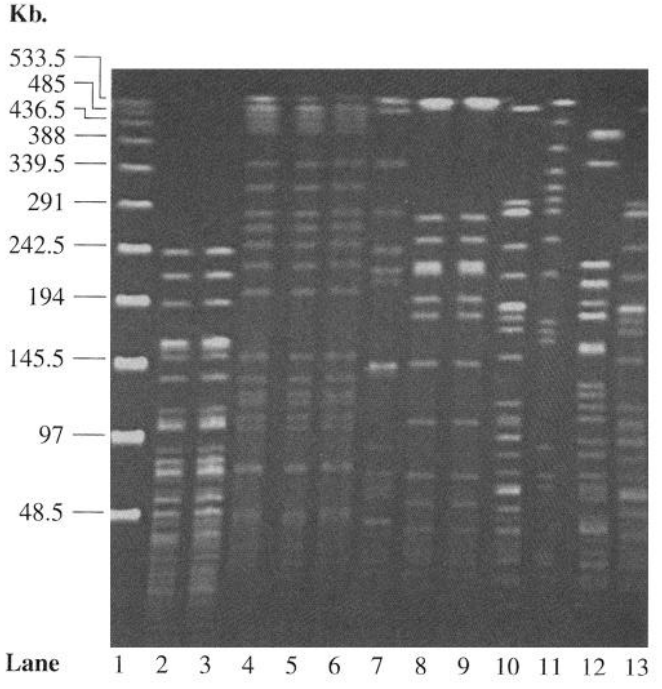

Fig. 2. PFGE of *Xba*I chromosomal digests from various Gram-negative species. Lane 1, Lambda concatamer; Lanes 2 and 3, *Pseudomonas aeruginosa*; Lanes 4–7, *Klebsiella* spp.; Lanes 8–13, *Enterobacter* spp.

The main advance in PFGE was the production of a homogeneous electric field by the use of multiple electrodes and differential voltages ***(4)***. The equipment for CHEF uses 24 electrodes arranged in a hexagon with the electric field intersecting at an angle of 120° in all parts of the gel. This gives rise to well-focused bands in straight lanes with DNA in all parts of the gel being subjected to the same conditions (**Fig. 2**). A further advance, programmable autonomously controlled electrodes (PACE), provides computer linkage to the CHEF apparatus and gives enhanced versatility to the system. This allows variable angles of intersection, multiple electric fields, different voltages, and a combination of programs within a single run. This system is marketed as the CHEF Mapper by Bio-Rad Laboratories (Richmond, CA) and as the Gene Navigator by Pharmacia Biotech (Uppsala, Sweden).

1.1. Parameters Affecting DNA Separation

Each aspect of the method will influence the final result and great care, therefore, needs to be given to the standardization of the method if gel-to-gel comparisons of banding patterns are to be made.

1 1.1. DNA Quality and Concentration

The main advantage of PFGE is its ability to separate large fragments of DNA following the restriction of unsheared chromosomes with rare-cutting endonucleases. DNA extracted as an aqueous solution for conventional electrophoresis is subjected to mechanical shearing resulting in DNA fragments of <500 kb—which is smaller than many of the fragments generated by rare-cutters—it is, therefore, not suitable for PFGE. Unsheared DNA is achieved by the incorporation of bacterial cells into agarose, followed by the lysis and disruption of the cells and the washing away of cellular debris.

The concentration of DNA affects its mobility and, hence, the clarity of bands and also the ease with which comparisons of banding patterns can be achieved both by eye and by computer-assisted analysis. DNA concentrations over 70 µg/mL reduce mobility and bands become increasingly diffuse. It is not possible to estimate the concentration of DNA embedded in agarose, so the concentration of cells embedded has to be carefully controlled to give the required end result.

1 1.2. Agarose Concentration

As in conventional gel electrophoresis, increases in agarose concentration retard the mobility of DNA fragments. For most PFGE applications, a concentration of 1% (w/v) is recommended, although increased resolution can be achieved with concentrations of 1.2–1.5% (w/v), whereas to separate fragments over 3 Mb, lower percentage gels are necessary, with a corresponding reduction in the clarity of bands. The quality of agarose used will produce noticeable differences in the mobility of fragments larger than 2 Mb. It is usually recommended that special PFGE agarose is used for these very large fragments, whereas molecular grade agarose can be used for those smaller than 2 Mb

1.1.3. Voltage and Pulse Times

In general, the mobility of DNA fragments smaller than 2 Mb increases with field strength, but this may be accompanied by a reduction in band clarity. The voltage most commonly used in PFGE is 6 V/cm distance between electrodes, whereas lower voltages, 2 V/cm, are required to separate DNA fragments over 2–3 Mb.

The most important parameter influencing the separation of large DNA fragments is the switching (or pulse) time. Simplistically, as pulse times increase, so does the size of DNA fragments that can be separated, provided that all other conditions remain the same. It is usual to ramp (change linearly) the pulse times from short to long during a run to increase the effectiveness of DNA separation. State of the art equipment allows the rate of ramping to be nonlin-

ear, thereby improving the separation of the lower or higher molecular weight fragments and by combining periods with different ramp ratios, almost any range of fragment sizes can be spread out to optimize and facilitate analysis. If a wide range of fragment sizes is expected, it may be necessary to divide the run into several different periods, varying the voltage applied as well as the range of pulse times.

1 1.4. Buffer Strength and Temperature

The mobility of DNA fragments through agarose increases with temperature, with large fragments being more susceptible to this influence than small fragments. Conversely, the clarity of focused bands decreases as the temperature rises. It is important to obtain a balance between speed of separation and clarity of banding patterns, and it is generally accepted that a realistic optimum temperature for PFGE is 14°C. Electrophoresis at 6 V/cm will generate a considerable amount of heat that, without active cooling, will raise the buffer temperature to an unacceptable level. Running equipment in a cold-room with the buffer circulating through ice will keep the temperature in the range of 10–14°C, but to maintain a constant monitored temperature, special cooling apparatus is required (available from most distributors of electrophoretic equipment) Reducing buffer concentration to 0.5X buffer instead of using 1X buffer also helps to reduce the generation of heat and to keep the temperature low. However, fragments move faster in 0 5X buffer necessitating a shorter run time.

Two buffers can be used for PFGE; Tris-borate EDTA buffer (TBE) and Tris-acetate EDTA buffer (TAE). In general, fragments move faster with TAE than with TBE, but the buffering capacity of TAE is lower, and for long runs the buffer may become exhausted before the completion of the run

1.2. Interpretation of Banding Patterns

Banding pattern interpretation will depend on the purpose of, and the question to be answered by, the PFGE. One of the most common uses of PFGE in medical microbiology is the comparison of epidemiologically related bacterial isolates in the investigation of outbreaks of infections in hospitals or in the community. Comparison of banding patterns by eye may be sufficient to decide which isolates are involved in the outbreak and which are different. To facilitate interpretation and to provide quantitation of the level of relatedness within and between groups of isolates, there are a number of computer programs available. These include GelCompar™ (Applied Maths, Kortrijk, Belgium), MVSP (Korvach Computing Services, Anglesey, UK), GelManager™ (BioSystematica, Tavistock, Devon, UK), Lane Manager™ (TDI), Phoretix 1D & 2D (Phoretix International Ltd., Newcastle, UK), and Dendron® (Solltech Inc., Oakdale, CA).

1.3. Applications of PFGE

1.3.1. Comparison of Epidemiologically Related Isolates

The main application of PFGE to clinical microbiology is in the determination of the relatedness of bacterial isolates from epidemiologic incidents. For some species, traditional typing schemes (serotyping, phage-typing, biotyping, and bacteriocin-typing) are available and, although of long standing, still provide a means of strain identification that is cheap to run, quick to provide, and reproducible. Reagents for these schemes are not, however, universally available nor are schemes available for all species. Chromosomal DNA is a fundamental molecule of all bacteria, and provides a focus for molecular methods that can be applied to all species. PFGE employs >80% of the chromosome to produce banding patterns, making it potentially the most discriminating method available today.

1.3.2. Investigation of Specific Regions of DNA Following Southern Blotting and Hybridization

The principles behind hybridization of PFGE gels are as described in Chapter 2. Difficulties can occur in the transfer of large DNA fragments and extra depurination incubation may be required. Transfer to nylon membrane takes longer for PFGE gels than conventional gels as they are thicker. Capillary blotting takes at least 24 h, whereas vacuum blotting takes about 4 h. Once the DNA has been transferred, the methods for hybridization are as described in Chapter 2.

1.3.3. The Separation and Sizing of Large Plasmids (5)

Large plasmids can be separated and sized by PFGE, without the removal of chromosomal DNA. Bacteria embedded in agarose are subjected to lysis followed by incubation with S1 nuclease, which converts supercoiled plasmids to full-length linear molecules. The migration of linear plasmid DNA is consistent with commonly used molecular weight markers allowing accurate plasmid sizing.

1.3.4. Physical Chromosome Mapping by Two-Dimensional PFGE (6)

Two-dimensional PFGE (2-D PFGE), in which partially digested DNA fragments are separated in the first direction followed by completion of digestion and separation of all fragments in the second direction, allows assembly of a chromosomal map based on the fact that bands liberated from a single partial band must be adjacent to each other on the chromosome. 2-D PFGE in which completely digested DNA is separated in the first direction, followed by complete digestion with a second enzyme in the second direction, can also be used to produce a genomic map. However, hybridization with known fragments may be required to complete the map by this method.

1.3.5. Comparison of Yeast Isolates

Methods for the preparation of DNA from yeast cells have been described *(7)* and used successfully to produce clear banding patterns. A word of caution has been expressed by Bostock et al. *(8)* when comparing *Candida albicans* isolates, however, as the high level of genetic switching that is characteristic of this species may result in two genetically similar isolates appearing different because of genetic rearrangement.

2. Materials

2.1. Preparation of Unsheared DNA

2.1.1. Incorporation of Bacteria into Agarose

1. Bacterial culture.
2. Low-gelling agarose.
3. Casting mold
4. SE buffer: 75 m*M* NaCl, 25 m*M* EDTA Na_2 (filter sterilized) Store at 4°C
5. Waterbath at 50–56°C.

2.1.2. Lysis of Bacteria

1. Appropriate lysis buffer
   Gram-negative bacteria. 1% (w/v) *N*-lauroyl sarcosine, 500 m*M* EDTA Na_2 pH 9 5 (*see* **Note 1**) Filter sterilize and store at room temperature. *See* **Table 1** for alternative lysis buffers
   Gram-positive bacteria 6 m*M* Tris-HCl, 100 m*M* EDTA Na_2, 1 *M* NaCl, 0 5% (w/v) Brij 58, 0.2% (w/v) sodium deoxycholate, 0 5% (w/v) lauroyl sarcosine, pH 7.5, stored at room temperature (*see* **Table 1**).
2. Proteinase K (Sigma, St Louis, MO, P-0390) Stock solution 50 mg/mL, stored at –20°C
3. Lysostaphin (for staphylococci only). For best results, using lysostaphin from Aplin and Barrett Ltd, Trowbridge, Wilts, UK, is recommended Stored at 4°C as 3000 U/mL in 50 m*M* Tris-HCl, 150 m*M* NaCl, pH 7 5
4. Lysozyme (*Acinetobacter* spp. and Gram-positive bacteria only). Store as powder at –20°C and add the appropriate amount to lysis buffer when required.
5. RNase. Stored at –20°C
6. Proteolysis buffer for Gram-positive bacteria (same composition as Gram-negative lysis buffer, *see* **step 1**, above)
7. TE buffer 10 m*M* Tris-HCl, 10 m*M* EDTA Na_2, pH 7 5 Filter sterilize and store at 4°C

2.2. DNA Restriction

1. Rare-cutting restriction endonuclease (*see* **Note 2**)
2. 10X reaction buffer (usually supplied with endonuclease)
3. Sterile distilled water
4. Agarose block with incorporated DNA

Table 1
Lysis Buffers used in PFGE

Species	Lysis buffer	Additives	Reference
Staphylococcus aureus	6 m*M* Tris-HCl, 100 m*M* EDTA Na_2, 1 *M* NaCl, 0.5% Brij-58, 0 2% sodium deoxycholate, 0 5% lauroyl sarcosine, pH 7.5	30–50 U/mL lyzostaphin 500 µg/mL lysozyme	***9***
Coagulase negative staphylococci	As for *S aureus*	As for *S aureus*, may need to increase lysozyme concentration	Author's unpublished observations
Micrococci	As for *S aureus*	2 mg/mL lysozyme	Author's unpublished observations
Enterococci	As for *S aureus*	1 mg/mL lysozyme 20 µg/mL RNase	***10***
Listeria monocytogenes	100 m*M* EDTA Na_2, 1% Sarcosyl	2 mg/mL deoxycholic acid 2 5 mg/mL lysozyme	***11***
Mycobacterium tuberculosis	Incubate in 10 m*M* Tris-HCl, 1 m*M* EDTA Na_2 prior to incorporation into agarose plug	2 mg/mL lysozyme	***12***
Bordetella pertussis	As for *S aureus*	1 mg/mL lysozyme 20 mg/mL RNase	***13***
Acinetobacter spp	May require *S aureus* lysis buffer	1 mg/mL lysozyme	Author's unpublished observations

2.3. Separation of DNA Fragments

1. Electrophoresis tank, power source, and switching unit suitable for PFGE (*see* **Subheading 1.**)
2. Cooling apparatus (*see* **Subheading 1.1.4.**).
3. Gel cassette and comb (*see* **Note 3**).
4. UV transilluminator and camera or gel documentation system. *Note* UV-protective face masks should be worn when working with a UV transilluminator as UV radiation can seriously damage the cornea and activate *Herpes simplex*
5. Molecular grade agarose
6. 0 5X TBE buffer: 44 5 m*M* Tris base, 44.5 m*M* boric acid, 1 m*M* EDTA Na_2, pH 8.0–8 5 Store as 5X TBE stock solution at room temperature The pH should not require adjustment.
7. Agarose block containing restricted DNA
8. Molecular weight marker (e g , lambda phage concatamers, *Escherichia coli* MG1655 digested with *NotI **[10]***).
9. 1 µg/mL ethidium bromide (EB). Store as 10 mg/mL aqueous solution at room temperature *Note* EB is a mutagen and carcinogen, avoid skin contact and inhalation and discard as toxic waste

3. Methods

3.1. Preparation of Unsheared DNA

Gloves should be worn at all stages of the method

3.1.1. Incorporation of DNA into Agarose

1. From plate cultures suspend sufficient growth in 1 mL of SE buffer to give an optical density of McFarland's tube 4 (*see* **Note 4**)
2. Prepare 2% low gelling agarose (LGA) in SE buffer. Dissolve by boiling and keep at 50–56°C until ready to use.
3. Assemble the mold
4. Warm the bacterial suspensions to 50–56°C and allow any clumps to settle
5. Mix 500 µL of LGA with an equal volume of bacterial suspension, mix gently but thoroughly. Do not vortex
6. Dispense the mixture into the mold and allow to solidify at 4°C. Many blocks can be made per isolate as, following lysis, blocks can be kept for many months in TE buffer provided the buffer is changed regularly

3.1.2. Lysis of Bacteria

3.1.2.1. Gram-Negative Bacteria

1. Prepare Gram-negative lysis buffer containing 500 µg/mL proteinase K.
2. Dispense sufficient lysis buffer into bijoux to cover the number of blocks made per isolate (2–3 mL is sufficient for five blocks)
3. Incubate overnight at 56°C (*see* **Note 5**).

4 Wash three times for 30 min each in TE buffer at 4°C
5 Store the blocks in TE buffer at 4°C.

3.1.2.2 Gram-Positive Bacteria

A more rigorous lysis is required to disrupt the Gram-positive bacterial cell wall, and different chemicals have been recommended in the literature for different genera. Some recommended cocktails are shown in **Table 1**

1 Prepare the appropriate Gram-positive lysis buffer
2. Dispense sufficient lysis buffer into bijoux to cover the number of blocks made per isolate (2–3 mL is sufficient for five blocks)
3 Incubate at 37°C overnight (*see* **Note 5**)
4 Replace the lysis buffer with proteolysis buffer containing 100 µg/mL proteinase K
5 Incubate at 56°C overnight
6 Wash and store the blocks as described for Gram-negative bacteria

3.2. DNA Restriction

1. Cut a portion of an agarose block containing DNA and transfer to a 500 µL reaction tube
2 Cover with 100 µL of 1X reaction buffer and keep at 4°C for 30 min
3 Replace reaction buffer with 100 µL of 1X reaction buffer containing 20 U of restriction endonuclease
4 Incubate at the temperature recommended by manufacturer for 4 h (*see* **Note 2**).

3.3. Separation of DNA Fragments

1 Prepare a 1–1.2% agarose gel in 0 5X TBE buffer. Dissolve agarose by boiling, allow to cool to 60°C before pouring into a gel-forming cassette (*see* **Note 6**) with a comb for wells in position Keep 5 mL of agarose at 56°C for sealing wells.
2 Leave the gel for at least 30 min to set Remove the comb and carefully load the agarose plugs containing digested DNA into the wells, ensuring that they are lined up against the leading edge of each well (*see* **Note 3**) If banding patterns are to be analyzed by computer, load molecular weight markers in every fourth, fifth, or sixth well
3 Seal each well with the reserved agarose, making sure that no bubbles are trapped
4 Remove the gel from cassette and place into the electrophoresis tank Secure the gel in position (*see* **Note 7**)
5 Cover the gel with 0 5X TBE (usually about 2 L) and allow to cool for at least 30 min by circulating the buffer through the cooling apparatus
6 Set the required parameters for electrophoresis (*see* **Note 8**)
7 When electrophoresis is complete, remove the gel from the tank and stain in EB for 1 h
8. Discard the tank buffer as it cannot be reused.
9 Destain the gel in distilled water for 1–2 h, changing the water every 30 min

10. Visualize the banding patterns under UV transillumination Keep a permanent record of the gel for analysis (*see* **Subheading 3.4.**).
11 Discard gels as toxic waste

3.4. Interpretation of Banding Patterns

3 4.1. Visual Interpretation

The level of analysis required to interpret banding patterns is in part dependent on the question to be answered. It is often possible to group isolates giving identical or very similar banding patterns and to distinguish between markedly different profiles at a glance, thereby determining quickly whether an outbreak is represented, whether cross-infection has occurred, or whether there is no sharing of isolates between patients.

Determination of the number of band differences between banding patterns of isolates from suspected outbreaks of infection has been advocated by Tenover et al. *(14)* for the interpretation of profiles having more than 10 bands. Each difference, indicated by the presence or absence of a band, is considered to be significant and four categories of relatedness have been proposed:

1. Indistinguishable, no band differences,
2 Closely related, two to three band differences;
3. Possibly related, four to six band differences; and
4 Different, seven or more band differences.

It should be noted that the larger the number of bands present, the more likely one is to see changes in the banding patterns, and although these criteria hold true for many species, some produce more variation within a strain than these criteria suggest, whereas others may be more clonal. Some authors equate a single band difference with the acquisition or loss of DNA by a fragment and its consequent apparent change in position because of change in molecular size *(15)*. However, this situation is considered by Tenover et al. *(14)* to represent two band differences. If band differences are to be used as the criteria for clustering and differentiating between isolates, it is advisable to consider first the number of bands found between strains, and then see how many bands need to be accommodated within a strain. Whereas this number will usually be within the criteria set down by Tenover et al. *(14)*, this will not always be so, and one will gain an understanding of the diversity to be expected within different species.

3.4.2. Computer Analysis

Many computer-assisted analysis packages are now available. These range in sophistication from those where the presence and absence of bands have to be keyed in for analysis (e.g., MVSP) to those employing image files produced from scanned photographs or from gel documentation systems. Such analysis

packages calculate coefficients of similarity and represent these as dendrograms (usually using unweighted pair group matching by an arithmetic averages algorithm, UPGMA, **Fig. 3**), similarity matrices, or evolutionary trees. Two commonly used coefficients are the Jaccard and Dice coefficients, both of which use the number of bands in common between profiles, and the total number of possible band positions to calculate the percentage similarity between the isolates. The formula for the Jaccard coefficient is.

$$n_{AB}/n_A + n_B - n_{AB}$$

where n_A and n_B are the numbers of bands in the profiles of isolates A and B, and n_{AB} is the number of shared bands. The Dice coefficient gives more weight to matching bands and is represented as:

$$2n_{AB}/n_A + n_B$$

A third coefficient offered by analysis packages is Pearson's correlation coefficient. One advantage of this calculation is that the whole densitometric trace is employed and specific band positions do not have to be defined. However, the quality of banding patterns affects the calculation, with variations in background staining being included. Hence, isolates with identical band positions that calculate as 100% similar by Jaccard or Dice coefficients may be calculated as only 80–90% similar by Pearson's coefficient. Irrespective of the coefficient used to calculate percentage similarity, the interpretation of the data has to be related to the epidemiologic situation when deciding which isolates are representatives of the "outbreak" strain and which are not. Figures of 80–85% similarity have been quoted to define a strain ***(15,16)***. However, just as the rules for number of band differences are not always applicable, so too do rules of percentage similarity sometimes fall down. It is, however, usual to find that there is a definite gap (more than 20%) between the percentage at which similar isolates cluster and the percentage at which isolates are clearly different, with only a few isolates falling between the two.

3.5. Troubleshooting

It is not uncommon for an electrophoretic procedure to be followed faithfully without obtaining the excellent results that one would expect. In techniques like PFGE, which are lengthy and contain many steps, it may be only when looking expectantly at the stained gel that one realizes that something has gone wrong, and it is often difficult to determine at what stage error occurred. This section contains some of the more common problems that may occur and details how to detect the cause.

1 There are faint bands, diffuse bands, or no bands present on the gel.
   a A few species of bacteria produce endogenous endonucleases that will

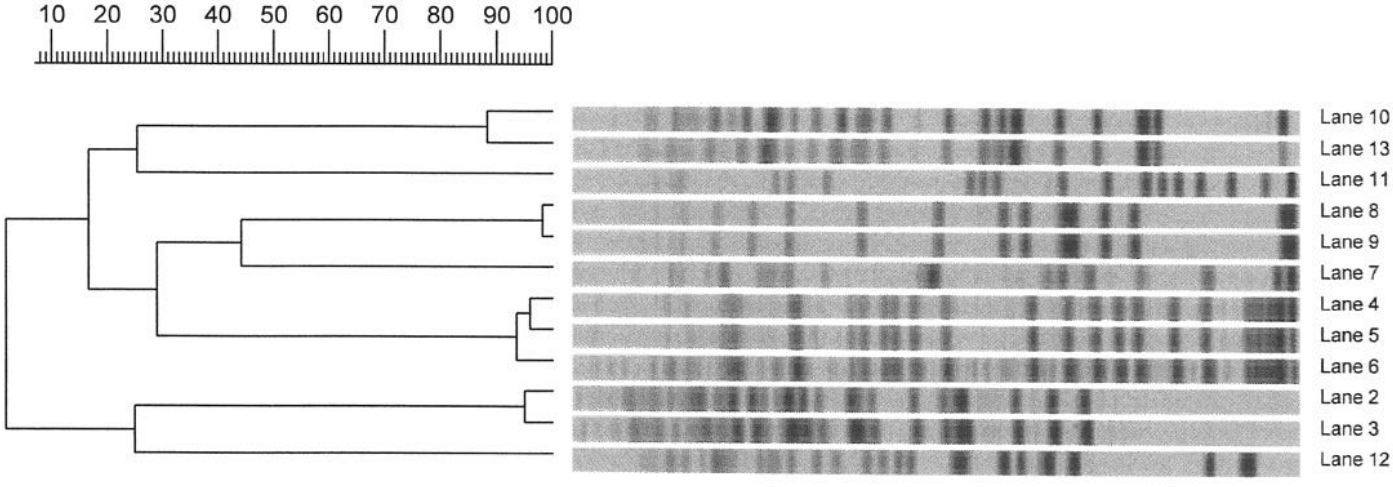

Fig. 3. Dendrogram of percentage relatedness of isolates in Fig. 2, as calculated by Pearson's correlation coefficient represented by UPGMA.

degrade digested DNA into fragments which will pass through the gel during PFGE Incubating the bacterial suspension in 10% formalin for 1 h followed by three washes in saline prior to incorporation in agarose has been found to inhibit DNase activity *(17)*

b Some Gram-negative bacteria lyse if kept at 56°C for a long time (more than 30 min) prior to incorporation into agarose. This may result in shearing of DNA and a reduction in DNA concentration to a level that produces faint bands, diffuse bands, or no bands If the DNA concentration is too high, bands may appear to bulge and not be clearly separated

c DNA digestion at an inappropriate temperature may result in incomplete or no restriction As a result, a single heavy band of DNA, a smear of DNA running the length of the lane, or DNA only in the wells may be seen

d The parameters set for the electrophoresis may be inappropriate for the sizes of fragments present (*see* **Note 8**).

e The concentration and temperature of the running buffer affects the mobility of DNA fragments If buffer concentration is high, the buffer temperature will rise during electrophoresis and may result in unresolved bands

f If the buffer circulation pump is set too fast, the passage of buffer over the gel can cause the gel to vibrate, which results in fuzzy bands Similarly, if the gel is not firmly secured before buffer is added, the gel may float either totally or partially with the result that bands are not well-defined

g EB that has been used to stain PFGE gels more than three times becomes too dilute and will fail to bind sufficiently with DNA to be visualized Conversely, bands are sometimes obscured by excessive background staining when using freshly made EB Extra washing in distilled water with gentle shaking will remove unbound EB and bands can be visualized

2. Bands are well-defined, but the lanes are bent, are of unequal length, or appear to be double.

a Provided the gel has not moved during the run, the most likely cause of lane distortion is that one or more of the electrodes has broken during electrophoresis Electrodes are subject to wear and tear as a result of the continuous switching of field direction, and the life of electrodes is considerably reduced if pulse times shorter than 5 s are used.

b The hour glass-like appearance of lanes that sometimes occurs can be reduced by equilibrating the gel in cold tank buffer for longer than suggested in **Subheading 3.3.**

c Bubbles accumulating under the gel during electrophoresis will result in differential cooling across the gel, which will cause the lanes in the center of the gel to be bowed, whereas the outer lanes remain straight

d. If the agarose block containing digested DNA breaks on loading, the DNA of each portion will run as separate lanes, with a gap between

3. Bands are present, but are not evenly spaced along the gel lane.

This is usually because the parameters used are inappropriate for the range of fragment sizes to be separated Some banding patterns have a high concentra-

tion of bands within a small range of fragment sizes It may be necessary to apply nonlinear ramping (CHEF mapper) or a series of linked short runs (CHEF DR-II or DR-III) to open up areas of dense bands.

4 Bands are very distorted
   a. It is very important that bacterial suspensions are uniform for incorporation into agarose. Some species aggregate or clump in suspension and, if these are incorporated into agarose, variable densities of cells will be present, and lysis will be inefficient If lysis is achieved, DNA concentrations will not be uniform and DNA mobilities will vary across the lane causing distortion
5 The stained gel appears speckled.
   a. Laboratory gloves may contain powder that fluoresces under UV transillumination. This will be detected by the photographic equipment Analysis of speckly gels is very difficult by eye and nearly impossible by computer
   b Agarose that has not been boiled for long enough and that is, therefore, not fully dissolved will have visible specks within the matrix. These will appear as spots in photographs

4. Notes

1 To achieve the required pH, it is necessary to add approx 10 mL of 10 *M* NaOH per 100 mL of lysis buffer before adjusting the pH dropwise
2 The choice of a rare-cutting endonuclease, which is an enzyme which recognizes DNA sites occurring infrequently in the genome, is dependent on the G + C ratio of the species under investigation, and the recognition site of the enzyme. Complex mathematical formulae are available for predicting the cutting frequency of an endonuclease *(18)*, but as a general rule enzymes recognizing mostly G and C will be rare cutters of AT-rich genomes and vice versa Longer recognition sites will tend to appear less frequently in the chromosome Endonucleases that recognize target sites that include the sequence CTAG are also usually rare cutters (**Table 2**). The majority of restriction endonucleases require incubation at 37°C, however, there are some that have different requirements. The length of time that an incubated endonuclease remains active varies considerably, and for some it may be necessary to use two periods of 2-h incubation with fresh buffer, rather than a single 4-h incubation.
3. It is important that the agarose blocks containing digested DNA are loaded carefully into the wells Broken inserts will distort the banding pattern If some inserts are loaded onto the leading edge and others are placed on the back edge of the well, identical banding patterns will not align. An alternative to loading inserts into wells is to place them onto the well-forming comb and carefully pour the gel around them. Once the gel has set, the comb can be removed and the holes filled with cool molten agarose It is worth practicing this method before using it for an investigation because the inserts will swim away from the comb if the gel is poured too rapidly.
4. Some bacterial species grow better in liquid culture. Optical density readings of liquid cultures can be calibrated to viable counts for estimation of cell concentra-

Table 2
Commonly used Rare-Cutting Restriction Endonucleases

Restriction endonuclease	Recognition site	Bacterial groups for which it acts as a rare cutter
*Apa*I	GGGCC↓C	*Acinetobacter* spp , Enterococci, *Listeria monocytogenes, Streptococcus* spp
*Asn*I	AT↓TAAT	Enterobacteriaceae, "Pseudomonads," *Mycobacterium* spp., *Bordetella pertussis.*
*Dra*I	TTT↓AAA	Enterobacteriaceae, "Pseudomonads," *Mycobacterium* spp , *Bordetella pertussis*
*Not*I	GC↓GGCCGC	*Escherichia coli* MG1655, *Staphylococcus aureus, Listeria monocytogenes*
*Sma*I	CCC↓GGG	*Staphylococcus aureus*, Enterococci, coagulase-negative staphylococci, *Listeria monocytogenes, Streptococcus* spp
*Spe*I	A↓CTAGT	Enterobacteriaceae, "Pseudomonads," *Mycobacterium* spp , *Bordetella pertussis*
*Ssp*I	AAT↓ATT	Enterobacteriaceae, "Pseudomonads," *Mycobacterium* spp , *Bordetella pertussis*
*Sst*II	CCGC↓GG	*Staphylococcus aureus*, coagulase negative-staphylococci
*Xba*I	T↓CTAGA	Enterobacteriaceae, "Pseudomonads," *Mycobacterium* spp, *Bordetella pertussis*

tion However, following reading, the cells need to be spun and washed three times before resuspending in SE buffer and incorporation into agarose. This may result in the loss of and disruption of cells, with corresponding changes in DNA concentration.

5 Many authors have published faster lysis regimens For example, Lindhardt et al *(19)* incubated *Staphylococcus aureus* and coagulase-negative staphylococci for 1 h in lysis buffer followed by 2 h in proteolysis buffer If speed of result is the driving force, then a quick lysis step is indicated However, from personal experience, the quality of the end product may not be guaranteed

6 Commercially available tanks are supplied with gel-casting molds. It is possible to run gels of any size that will fit within the arrangement of the electrodes With the largest possible gel sizes, some lane distortion may occur in the extremities.

7 During electrophoresis, buffer is circulated to maintain an optimum temperature If the gel is not firmly secured, it will float and move with the current resulting in distortion of bands or no bands at all Most commercial tanks are supplied with a means of anchoring compatible gels. If gels of different sizes are used, it may be necessary to secure these with small pieces of plastic (e g , cut off ends of tips or reaction tubes) and waterproof tape

8 Electrophoresis parameters that produce the best separation of banding patterns will need to be found empirically Some general guidelines can, however, be given
   a In general, short switching times separate small fragments, whereas longer switches are required to separate larger fragments
   b Longer gels require longer run times (e g., banding patterns that spread evenly along the lane in a 15-cm gel in 18 h, may need 30–35 h in a 20 cm gel and 40–48 h in a 25-cm gel)
   c Composite runs may be required to separate fragments clearly if fragments differ only slightly in size.
   d. A useful set of conditions from which to start experimenting are

Initial pulse	5 s
Final pulse	35 s
Ratio	1 1
Run time	18 h for 15-cm gel

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